

Regulated Lentiviral Packaging Cell Line Devoid of Most Viral *cis*-Acting Sequences

Malvika Kaul,^{*,†} Hong Yu,^{*,1} Yacov Ron,^{*} and Joseph P. Dougherty^{*,2}

^{*}Department of Molecular Genetics and Microbiology, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, New Jersey 08854; and

[†]Graduate Program in Microbiology and Molecular Genetics, Rutgers University, New Brunswick, New Jersey 08903

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Packaging cell lines derived from human immunodeficiency virus-1 (HIV-1) are promising tools for *in vivo* somatic cell gene therapy protocols due to the ability of lentiviruses to infect nondividing cells. We describe here the generation of a safe, stable HIV-1 packaging cell line capable of expressing all of the HIV-1 structural, enzymatic, and regulatory proteins but lacking majority of the *cis*-acting sequences. The use of an inducible expression system circumvented the cytotoxic and cytostatic effects associated with the expression of some of the HIV-1 viral proteins. Reverse transcriptase activity was detectable in the supernatant from the stable packaging line 1 day after induction, while vector titers peaked 5 days postinduction. Vector titers of up to 3.5×10^4 infectious units/ml (IU/ml) were maintained through 8 months following the establishment of the cell line. Lineage-specific gene delivery can be achieved from this established cell line as viral stocks obtained specifically infect CD4⁺ target cells. Moreover, this cell line provides a safe and easy to use system for screening of drugs that inhibit HIV-1 replication. © 1998 Academic Press

INTRODUCTION

A hallmark of retrovirus replication is the very efficient integration of the viral DNA into the genome of an infected cell, where it is stably maintained and transmitted to daughter cells. This characteristic has provided impetus for developing retroviral vector systems for the efficient transduction and the long-term maintenance and expression of exogenous genes in primary cells. There are two basic components of a retroviral vector system, the defective vector virus containing the *cis*-acting sequences required for efficient viral replication and the packaging cell, which provides the viral proteins required in *trans* for viral replication. Most retroviral-based vectors described to date have been derived from the oncoretrovirus, murine leukemia virus (MLV) (Harris and Lemoine, 1996; Robbins *et al.*, 1998). While MLV-based vectors have proven useful for *ex vivo* transduction, a limitation of these systems is that proviral integration into the host genome requires at least one round of cell division (Miller *et al.*, 1990). Nuclear envelope breakdown that accompanies cell division appears to be necessary for the nuclear import of the oncoretroviral preintegration complex to permit the integration process (Lewis and Emerman, 1994). In contrast, the preintegration complex of the lentivirus human immunodeficiency virus-1 (HIV-1)

can directly traverse the nuclear envelope barrier, reportedly due to the nuclear localizing sequences present within the HIV-1 proteins Vpr (Heinzinger *et al.*, 1994), MA (Gallay *et al.*, 1995a), and IN (Gallay *et al.*, 1995b). This ability to infect nondividing cells provides an incentive for the development of HIV-1 based packaging cell systems for use with *in vivo* gene therapy. Additionally, HIV-1 packaging lines would also be valuable for studies of lentiviral biology. For example, such cell lines allow one to establish assays that can be utilized to study HIV-1 replication, mutation frequencies, and latency (Yu *et al.*, 1997). Moreover, inducible packaging cells provide a safe system for drug screening since viral proteins are not produced until induction and biologically hazardous wild-type virus is never generated.

Recently several reports detailing gene transfer into nondividing cells using lentiviral shuttle vectors have been described (Naldini *et al.*, 1996a,b; Aiken, 1997; Bartz and Vodicka, 1997; Blömer *et al.*, 1997). However, most employ transient protocols to produce the vector stocks, which can vary greatly with each transfection, and have the potential for untoward homologous and nonhomologous recombination events. These drawbacks with transient transfection procedures limit their application in basic and clinical research.

Most of the transient protocols published to date describe the generation of HIV-1 particles pseudotyped with a heterologous envelope, the vesicular stomatitis virus G protein, VSV-G (Naldini *et al.*, 1996a,b; Blömer *et al.*, 1997). The use of VSV-G has helped to bolster titers to levels which are desirable for efficient gene transfer into primary cells but concomitantly results in loss of target

¹ Present address: Section of Immunobiology, Howard Hughes Medical Institute, Yale University School of Medicine, 310 Cedar Street, New Haven, CT, 06510.

² To whom correspondence and reprint requests should be addressed. Fax: (732) 235-5223. E-mail: doughejp@umdnj.edu.

cell specificity. Stable HIV-1 packaging lines which maintain the target cell tropism afforded by the HIV-1 envelope would have the potential to more reproducibly yield lineage-specific viral stocks capable of infecting nondividing cells. HIV-1-based vectors may be particularly useful in the treatment of HIV-1 infection, as the antiviral exogenous gene would be specifically targeted to the HIV-1 host cells. Moreover, the vector virus could also be rescued *in vivo* by the patients' wild-type virions, and thus comparatively lower titers of the therapeutic vector may be permissible as *in vivo* amplification is possible (Poeschla *et al.*, 1996).

Several biological features of HIV-1 make it challenging to successfully develop a safe, efficient HIV-1-based packaging line. First, the pathogenicity associated with the virus requires that the viral stocks be completely void of any replication competent virus. Also HIV-1 expresses several accessory proteins reported to enhance high-level infection/replication, and it would therefore be desirable to include them in the packaging cell line (Heinzinger *et al.*, 1994). However, a technical hurdle exists in that several of the *trans*-acting proteins, including Env (Sodroski *et al.*, 1986), Pro (Kaplan and Swanstrom, 1991), and Vpr (Bartz *et al.*, 1996) have been reported to be cytotoxic or cytostatic, hampering the establishment of a constitutively expressing packaging cell line.

Extending upon previous work by our laboratory in which a tetracycline inducible system was employed to establish a HIV-1 packaging cell line (Yu *et al.*, 1996), this paper reports the creation of an additional inducible cell line which, unlike the previous producer line, is capable of expressing all the regulatory proteins of HIV-1. Application of this line should lend greater authenticity to studies of HIV-1 biology (i.e., replication, recombination, latency, etc.) as all viral proteins are present, and hence this system mimics an *in vivo* HIV-1 infection more closely. To achieve a higher degree of safety, the packaging line described herein also lacks most of the 5' encapsidation sequences as well as the 5'LTR and part of the 3'LTR. Transduction titers of up to 3.5×10^4 IU/ml are reported.

RESULTS

Design of the tetracycline-inducible HIV-1 packaging cell line

Packaging cell lines are engineered to produce the *trans*-acting viral proteins. In our packaging cell line system, the expression of the *trans*-acting HIV-1 proteins is tightly regulated by two levels of induction. First, the early gene products, including Rev and Tat, are expressed from an inducible promoter consisting of the heptamerized tetracycline operator (*tetO*) sequences from *Escherichia coli* coupled to a minimal CMV promoter (Gossen and Bujard, 1992). Transcription from this promoter is upregulated only in the presence of the

transactivator, tTA, a fusion protein between the tetracycline repressor and the activation domain of the herpes simplex virus VP16 protein. When present, tetracycline binds to the tTA protein, resulting in allosteric changes in the transactivator such that it cannot bind to the *tetO* sequences, resulting in a suppression of viral protein expression. In the absence of tetracycline, tTA can bind to and transactivate the inducible promoter, leading to expression of downstream genes. Although transcription of all the HIV-1 genes is controlled by this promoter in our system (Fig. 1), the translation of the late gene products is possible only after Rev is produced, since the nuclear export of the singly spliced and unspliced RNA encoding these proteins is possible only in its presence. This results in two levels of induction, as expression of the late gene products, which include Gag, Pol, Env, Vpr, Vpu, and Vif, is possible only after expression of Rev, which is in turn dependent upon the absence of tetracycline.

To address safety concerns, the inducible packaging cell lines were generated by separating the *gag-pol* and *env* genes by splitting expression from two different recombinants. Additionally, *rev* expression was placed on the *env*-containing construct, effectively separating it from the *tat* gene (Fig. 1). Furthermore, the two expression plasmids were sequentially transfected into the packaging cell to reduce the possibility of recombination resulting in production of replication-competent virus (Markowitz *et al.*, 1988).

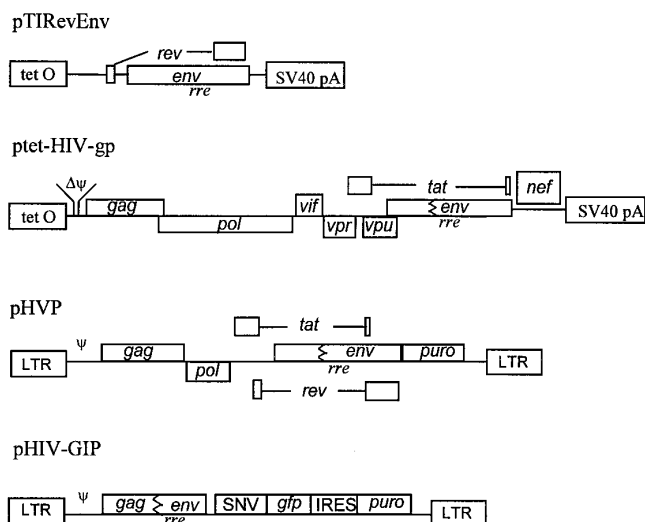


FIG. 1. HIV-1 packaging cell constructs and vectors. Boxes interrupted with jagged line symbolize partial deletions; $\Delta\Psi$ signifies a 33-bp deletion downstream of the splice donor site (nucleotide 295–328), which deletes at least part of the encapsidation signal; *tetO*, heptamerized tetracycline operator fused with a minimal CMV promoter (Gossen and Bujard, 1992); SV40 pA, SV40 polyadenylation signal; *puro*, puromycin gene; SNV spleen necrosis virus U3 promoter; *gfp*, green fluorescent protein gene; IRES, internal ribosomal entry site from encephalomyocarditis virus (EMCV) 5' nontranslated region (NTR) (Koo *et al.*, 1992).

Establishment of the HIV-1 packaging cell line

The generation of the parental Env-inducible cell line #69 has been described previously (Yu *et al.*, 1996). Briefly, pTIRvEnv was cotransfected along with the marker plasmid pHMR272 (Bernard *et al.*, 1985), expressing the hygromycin resistance gene, into HtTA-1 cells, a HeLa-based cell line that constitutively expresses the fusion protein tTA (Gossen and Bujard, 1992), and a stably transfected cell clone designated #69 was obtained (Yu *et al.*, 1996). #69 cells were then cotransfected with the ptet-HIV-gp (Fig. 1) and pSV2gpt constructs and selected for resistance to GPT (Mulligan and Berg, 1981). Sixty-eight clones were expanded and screened by fusion and reverse transcription assays. HeLa-CD4-LTR- β gal cells were used for the fusion assay (Kimpton and Emerman, 1992). These cells fuse with HIV-1 Env-expressing cells because they bear CD4 on their cell surface. HeLa-CD4-LTR- β gal also harbors a vector encoding the *lacZ* gene driven by the HIV-1 5'LTR; in the presence of the viral protein Tat, this promoter is transactivated leading to the expression of *lacZ*. By cocultivating HeLa-CD4-LTR- β gal with a putative packaging line, the expression of Tat can be ascertained in the line by staining the fused cells with Xgal. Thus this fusion assay screens for expression of proteins from plasmids, pTIRvEnv and ptet-HIV-gp in the cell. Ten clones positive for both fusion and RT activity were further characterized.

Inducible viral protein expression

To ensure that the viral proteins are expressed only upon the removal of tetracycline, the levels of the envelope glycoprotein, gp120, and matrix protein, p24 were tested prior to and after induction. Western blots are shown using total cell lysate from one of the clones selected, designated B16, before induction, and on Days 1–7 after induction (Figs. 2A and 2B). Neither protein was detectable before induction, but positive bands were clearly visible 5 days after induction. However, reverse transcriptase activity was detectable 1 day postinduction, with no activity detectable before induction (Fig. 3). One possibility to explain why gp120 and p24 were not detectable in cell lysates until 5 days postinduction in comparison to 1 day for the reverse transcriptase activity is that the latter is an enzymatic assay, which is more sensitive than Western blots. The sensitivity of the Western blots is also dependent on the quality of the antibodies used for detection.

Transduction titers of packaging cell line

The HIV-1-derived vector pHVP (Richardson *et al.*, 1993) was used to test the ability of cell lines to propagate vector virus (Fig. 1). Mass populations of the 10 packaging cell lines each stably transfected with pHVP

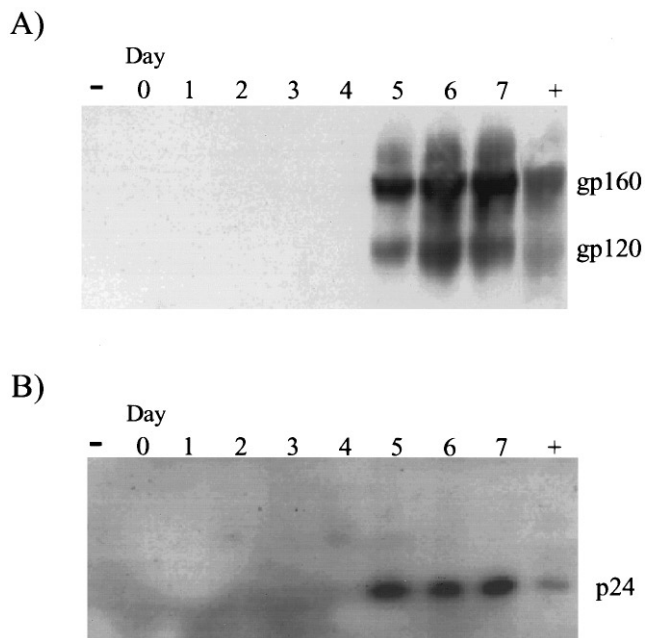


FIG. 2. Western blotting to analyze inducible expression of gp120 (A) and p24 (B). Total cell protein from the B16 cell line was extracted on Day 0 prior to induction and on Days 1–7 postinduction and used for Western blotting as described in Materials and Methods. +, cell lysate from HeLaT4 cells infected with wild-type HIV-1; –, cell lysate from uninfected HeLaT4 cells.

were obtained by selection with puromycin. The cells were then induced by tetracycline withdrawal, and supernatant was harvested each day for a week and used to inoculate HeLaT4 target cells. The target cells were placed under puromycin selection 24 h postinfection, and titers were calculated by counting the number of puro-resistant colonies 2 weeks later. Different lines gave peak titers on different days postinduction. Highest titers were obtained from two different lines, B16 and D11. pHVP-transfected B16 cells (B16HVP) yielded peak titers 5 days postinduction, ranging from 1.0×10^3 to 1.5×10^3 IU/ml (Table 1), while pHVP-transfected D11 cells yielded peak titers 4 days postinduction, ranging from 0.6×10^3 to 2.0×10^3 IU/ml. However, because the level of gp120 expression by clone D11 as detected by Western blots was comparatively low, B16 was chosen for further analysis.

Southern blotting was employed to confirm that the infected target cells contained the transduced vector. This was done by extracting genomic DNA from individually expanded puro-resistant HeLaT4 cells, which had been infected with viral stocks from B16HVP cells, and subjecting the DNA to Southern blotting. The blots were probed with a portion of the *gag* gene to detect a 2.2-kb fragment of the transduced vector (Fig. 4A); all progeny cell clones were verified to contain the vector provirus (Fig. 4B).

Transduction titers of clone B16 were confirmed with another HIV-1-based vector pHSGIP (Fig. 1). Harvested

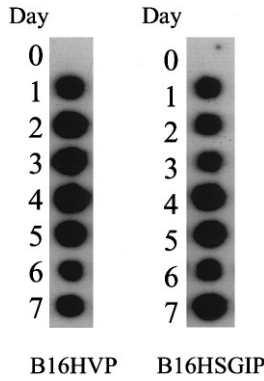


FIG. 3. Reverse transcriptase assay. Supernatant from B16 cells transfected with pHVP or pHIV-GIP was harvested on Day 0 prior to induction and on Days 1–7 postinduction and assayed for reverse transcriptase activity as described in Materials and Methods.

supernatant from pHSGIP transfected B16 cells (B16HSGIP) was used to infect target cells and titers scored by counting puromycin resistant colonies. These titers were also confirmed by counting the number of green fluorescent target cells clones. Stable B16HSGIP cells yielded titers ranging from 0.5×10^3 to 0.7×10^3 (Table 1). However, titers ranging from 2.0×10^4 to 3.5×10^4 IU/ml were obtained from a clone designated #2B16GIP, which was obtained by subcloning from the mass population of B16HIV-GIP cells (Table 1). Sub-clones often provide higher titers than the mass populations as they are representative of a homogenous set of cells.

Supernatant samples collected from clones B16HVP and #2B16GIP were also assayed for the presence of replication-competent virus as described previously (Yu *et al.*, 1996). Briefly, H9 cells were inoculated with the supernatant collected on Day 5 postinduction from the packaging lines, and cultured continually for 30 days.

TABLE 1

Transduction Titers from Packaging Cell Line B16

Day	Titer (IU/ml) ^a		
	B16HVP	B16HIV-GIP	#2B16GIP
1	1.0×10^1	ND	ND
2	1.6×10^1	ND	ND
3	3.0×10^1	ND	5.0×10^2
4	3.5×10^2	ND	1.6×10^3
5	$1.2 \pm 0.3 \times 10^3$	$0.6 \pm 0.1 \times 10^3$	$2.9 \pm 0.8 \times 10^4$
6	1.5×10^3	ND	$1.1 \pm 0.5 \times 10^4$
7	$1.1 \pm 0.4 \times 10^3$	$0.2 \pm 0.1 \times 10^3$	$0.5 \pm 0.2 \times 10^4$

^a Vector virus titers were determined by infecting HeLaT4 cells as described in Materials and Methods. Where standard deviations are reported, results represent average titers from three separate experiments. Otherwise, titers reported are from a single experiment. ND, not determined.

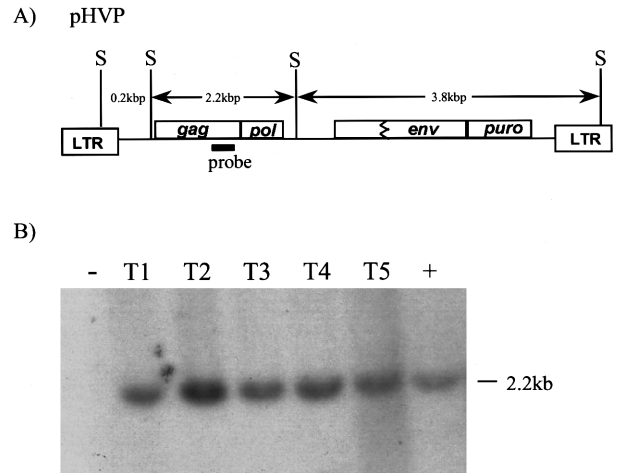


FIG. 4. Southern analysis for target cell transduction. Genomic DNA samples from individual HeLaT4 target cell clones, designated T1, T2, T3, T4, and T5 which had been infected with supernatant from pHVP-transfected B16 cells were digested with *SacI* (S), and subjected to Southern analysis. (A) Diagram of HIV-1 vector pHVP indicating the probe used from the gag region that detects a specific 2.2-kb fragment from *SacI* digested proviruses. (B) Southern blot showing diagnostic band of 2.2-kb band in all five clones. +, *SacI*-digested pHVP plasmid; –, genomic DNA from uninfected HeLaT4 cells.

Culture supernatant from the inoculated H9 cells were collected periodically over 30 days, and on the 30th day used to inoculate fresh H9 cells. Supernatant from the freshly inoculated cells was collected 5 days later and, along with the previously collected supernatant samples, assayed for the presence of p24^{gag} via ELISA. The levels of the antigen in the supernatant samples were indistinguishable from the background levels. The original supernatant from clone B16 used to inoculate the H9 cells and purified p24^{gag} antigen were used as positive controls. The results confirmed that under the assay conditions used, the packaging cell line B16 did not produce detectable levels of replication-competent virus.

DISCUSSION

This report describes the establishment of an inducible, safe, and stable HIV-1 packaging cell line, B16. Expression of all viral proteins is tightly regulated in B16 under the control of a tetracycline-responsive promoter. B16 was determined to have transduction titers of $2.9 \pm 0.8 \times 10^4$ IU/ml and not produce replication competent virus at detectable levels.

The packaging line, B16, completely lacks all viral sequences located upstream of nucleotide 253 of the HIV-1 genomic RNA, including the 5' LTR and the primer binding site (pbs); part of the 3' LTR has also been excluded. In addition, the *gag-pol* construct, ptetHIV-gp has a deletion between the 5' major splice donor site and the gag initiation codon (nucleotides 295–328) which corresponds to a predicted stem-loop structure report-

edly critical for RNA encapsidation (McBride and Panganiban, 1996). The combination of these deletions results in a lentiviral packaging cell line that lacks the majority of the *cis*-acting sequences considered important for HIV-1 virion packaging. Expression of HIV-1 proteins from two vectors, pTIREvEnv and ptet-HIV-gp, introduced sequentially affords an additional tier of safety by greatly reducing the probability of recombination during transfection which could contribute to the emergence of replication competent virus.

Additionally, ptet-HIV-gp contains open reading frames for all HIV-1 accessory proteins. As the biological roles of the HIV-1 regulatory proteins have yet to be defined precisely, this line may prove useful in protein function investigations. Furthermore, as B16 is a HeLa-based cell line, it does not express the HIV-1 Env receptor, CD4. Therefore the producer cell line cannot be infected with the vector virus it generates, and so there is no cycling of the vector virus once it is produced. Thus an assay involving a single cycle of replication of vector virus from producer cells to target cells can be established and be utilized for studies of HIV-1 mutation and recombination rates (Yu *et al.*, 1997). The packaging line also provides a model system to explore other aspects of HIV-1 biology including HIV-1 replication and latency.

Currently, the more common methods used for screening of anti-HIV-1 drugs *in vitro* is by detecting p24 HIV-1 core antigen production by infected cells or by HeLa-CD4 plaque assays (Gervaix *et al.*, 1997). Monitoring inhibitory effects of drugs to HIV-1 replication by these methods requires multiple manipulations and can be time consuming. The B16 packaging cell line can be used to investigate anti-HIV-1 drug susceptibility. A decline in viral titers by the action of putative therapeutic anti-HIV-1 drugs can be rapidly ascertained within 48 h by the use of a GFP-encoding vector such as pHSGIP. By performing the assay in 96-well plates, multiple drugs can be screened at a range of concentrations. As all the viral proteins in HIV-1 infected patients are present in this line, the antiviral activity of a potential drug can be accurately tested. Studies to examine such drugs have already been initiated in our laboratory (M.K. and J.D., unpublished data). Hence this packaging line provides a fast, easy, inexpensive, and safe system to screen for antiviral drugs.

Two groups have previously reported the construction of constitutive HIV-1 packaging cell lines with titers ranging from 10^3 to 10^5 IU/ml (Corbeau *et al.*, 1996; Poeschla *et al.*, 1996; Srinivasakumar *et al.*, 1997). However, it is unclear whether or not these cell lines express all of the accessory proteins. For instance, as mentioned earlier, Vpr has cytostatic properties in a variety of cell types (Bartz *et al.*, 1996), and in a constitutively expressing HIV-1 packaging cell line, the level of Vpr would therefore be expectedly low or nonexistent. This might not be reflected in titers

obtained with dividing cells but would perhaps dampen titers on nondividing cells, as Vpr has been implicated in conferring upon HIV-1 the ability to infect nondividing cells (Heinzinger *et al.*, 1994). It is possible, nevertheless, that proteins expressed specifically from the strains of HIV-1 which were used in these lines (HIV-1 MN and HIV-1 NL4-3) have different levels of toxicity. The inducible system we adopted for the packaging line described in this manuscript would circumvent the toxicity associated with constitutive expression of HIV-1 proteins and may have the additional advantage of synchronizing viral protein expression.

Transient HIV-1 packaging lines which produce HIV-1 particles pseudotyped with a heterologous envelope protein, VSV-G, have also been recently described (Naldini *et al.*, 1996a,b; Blomer *et al.*, 1997). Forming VSV-G pseudotyped vector virus presents the significant advantage for gene therapeutics of yielding high-titer viral stocks, as this envelope is less sensitive to concentration of the stocks by ultracentrifugation. However, even though VSV-G allows for concentration without appreciable loss of infectivity, the target cell tropism of VSV-G is too broad for many gene therapy applications. The use of the HIV-1 envelope permits specific targeting of cells in the CD4⁺ compartment. This would be especially useful in HIV-1-directed gene therapy, as the antiviral genes would be targeted to HIV-1-infected or -infectible host cells. The titers achieved by our line are too low for efficient *in vivo* gene transfer, and attempts are underway currently in the laboratory to boost titers. However, an increase in transgene dispersal may be possible utilizing low titers as HIV-1-based vectors could be rescued *in vivo* in infected patients by their wild-type virions and thus spread to a larger pool of target cells. The use of HIV-1 vectors to target viral RNA of infected cells with genes such as antisense RNAs and ribozymes ensures the intracellular colocalization of target and effector molecules. Inducible expression of an antiviral gene (e.g., suicide gene) may also be achieved by exploiting the dependence of HIV-1 gene expression upon the regulatory proteins Tat and Rev.

The generation of the B16 cell line is an advancement toward the development of lentiviral-based vectors for gene therapy. Although titers presently achievable of HIV-1 vectors in conjunction with the HIV-1 envelope are still 10-to 100-fold lower than those obtained with the murine retroviral systems, further improvement of the HIV-1 systems is desirable as the usefulness of the murine-based systems for *in vivo* therapy is limited. Apart from its application in HIV-1 gene therapy, the vector virus from a HIV-1 packaging line has the potential to transduce nondividing CD4⁺ lymphocytes, monocytes/macrophages, and glial cells *in vivo*. Long-term expression of a therapeutic gene in the hematopoietic compartment might thus be achieved.

MATERIALS AND METHODS

Plasmid construction

Plasmid ptet-HIV-gp was constructed from a proviral clone of the LAI strain of HIV-1 containing deletions in the encapsidation sequences from nucleotides 241–253 and 295–328, a gift from Dr. Julian J. O'Rear, UMDNJ-Robert Wood Johnson Medical School (numbers correspond to viral RNA nucleotide sequence) (Kim *et al.*, 1994). A *Bgl*II deletion between 6630 and 7215, preventing expression of the *env* gene, and a *Bam*HI-*Ssp*I deletion between 8068 and 8200, mutating the *rev* gene, were introduced; the entire segment initiating from the *Bss*HII site (256) to the *Sac*I site (9170) was moved under the transcriptional control of tetracycline-responsive inducible promoter between the *Eco*RI/*Bam*HI sites in pUHD10-3 (Gossen and Bujard, 1992). Thus ptet-HIV-gp contains open reading frames for Gag, Pol, Tat, Vpr, Vpu, Vif, and Nef but has mutations, deletions, or disruptions for the packaging signal (ψ), primer binding site (pbs), Rev, Env, and the 5' and 3' LTRs. pTIREvEnv and pHVP have been described previously (Richardson *et al.*, 1993; Yu *et al.*, 1996). The HIV-1 based retrovirus vector pHIV-GIP was constructed as follows: (1) a 5.8-kb deletion (961–6854) was created in the provirus clone pLAI3 (a gift from Dr. Michael Emerman) to generate p Δ LAI3; (2) the spleen necrosis virus (SNV) U3 promoter (Emerman and Temin, 1984) was inserted into p Δ LAI3 at the *Xho*I site; and (3) the green fluorescent protein gene (*gfp*) linked to the puromycin resistance gene (*puro*) via an internal ribosomal entry site (IRES) was obtained as a cassette (the GFP-IRES-*puro* cassette) from a modified version of pJZ442, a gift from Dr. Jiayou Zhang, by double digestion with *Bam*HI and *Nhe*I and placed under the transcriptional control of SNV U3 promoter (Koo *et al.*, 1992; Chalfie *et al.*, 1994). Further cloning details are available upon request.

Cell culture and generation of the packaging cell line

HeLa cells (Jones *et al.*, 1971) and 293T (DuBridge *et al.*, 1987) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The HeLaT4 cell line was a gift from Dr. Michael Emerman and was grown in DMEM supplemented with 10% FBS and 100 μ g/ml of hygromycin. HeLa-CD4-LTR- β gal cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. Michael Emerman (Kimpton and Emerman, 1992) and were grown in DMEM supplemented with 10% FBS, 100 μ g/ml hygromycin, and 200 μ g/ml G418. The inducible cell line #69 expressing Env and Rev (Yu *et al.*, 1996) was grown in DMEM with 10% FBS, 100 μ g/ml hygromycin, 200 μ g/ml G418, and 2 μ g/ml tetracycline.

To establish the inducible packaging cell line, #69

cells (Yu *et al.*, 1996) were cotransfected via the modified calcium phosphate precipitation method (Gorman, 1985) with 20 μ g of ptet-HIV-gp and 0.1 μ g of pSV2gpt, which expresses the *Escherichia coli* xanthine-guanine phosphoribosyltransferase gene, *gpt* (Mulligan and Berg, 1981). Transfected cells were selected for resistance to GPT (250 μ g/ml xanthine, 15 μ g/ml hypoxanthine, and 7 μ g/ml mycophenolic acid) in the presence of tetracycline (2 μ g/ml). Individual colonies were screened for HIV-1 Tat protein expression by fusion assay as follows. Cells were cocultivated with HeLa-CD4-LTR- β gal cells (Kimpton and Emerman, 1992) at a 1:1 ratio, with 2×10^4 total cells per well in a 24-well plate with or without tetracycline for 4 days, followed by X-gal staining (Kimpton and Emerman, 1992) and microscopic inspection to identify blue syncytia. Supernatant was collected during this time to assay for reverse transcriptase (RT) activity, which was carried out according to standard protocol (Willey *et al.*, 1988). Cell clones which exhibited blue syncytia with HeLa-CD4-LTR- β gal cells and were positive for RT activity upon induction were further analyzed.

Western blotting to test for inducible HIV-1 protein expression

Western blotting was employed to assay the putative packaging cells for inducible *gag* and *env* expression. Samples of the packaging cells were collected prior to, and on Days 1–7 after induction. Total cell protein was obtained by lysing the cells in buffer containing 20 mM Tris (pH 7.4), 5 mM $MgCl_2$, 0.1 M NaCl, 1% NP-40, 0.5% sodium dodecyl sulfate, and 1% aprotinin. Protein concentrations were determined using the BCA protein assay reagent (Pierce, Rockford, IL) as described by the manufacturer. Total cellular protein (100 μ g) from each sample was denatured, separated through either 7.5 or 15% SDS–polyacrylamide gels, and blotted onto polyvinylidene difluoride membrane (Dupont, Boston, MA). After blocking with 5% nonfat dry milk (Bio-Rad) in TBS-T [150 mM NaCl, 50 mM Tris (pH 7.4), 0.05% Tween 20] overnight at 4°C, the blots were incubated with sheep polyclonal anti-gp120 antiserum (Hatch *et al.*, 1992; Page *et al.*, 1992) or sheep polyclonal anti-p24 antiserum (Karacostas *et al.*, 1989) for 1 h at 1:50,000 or 1:1000 dilutions, respectively. Both antisera were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Michael Phelan. Unbound antiserum was removed from the blots by washing three times with TBS-T. Bound antiserum was treated with peroxidase-conjugated donkey anti-sheep antibody (Jackson ImmunoResearch Laboratories, Inc.) at a 1:50,000 dilution for 1 h at room temperature. The membranes were again washed three times with TBS-T, and bound conjugated antiserum was detected by using the Renaissance chemiluminescence reagent as described by the manufacturer (NEN, Dupont).

Virus production and infection

pHVP (20 μ g) was stably introduced into the putative packaging cells by the modified calcium phosphate precipitation method (Gorman, 1985), followed by selection with 1.0 μ g/ml puromycin in the presence of 2 μ g/ml tetracycline. To test the packaging capability, the cells containing pHVP vector were seeded at 1×10^6 cells per 100 mm dish in media containing tetracycline (2 μ g/ml) and were induced by withdrawal of tetracycline 24 h after seeding. Supernatant was harvested on Days 1–7 after induction, and titers were determined by the infection of HeLaT4 cells. HeLaT4 cells (3×10^5) were first treated with 2 μ g/ml of Polybrene for 30 min, followed by inoculation with 0.3 ml of supernatant at 10-fold serial dilutions for 2 h. Transduced cells were selected for puromycin resistance (1.0 μ g/ml) 24 h postinoculation. The pHIV-GIP vector was tested similarly. Additionally, the titers obtained from pHSGIP were confirmed by counting the number of green fluorescent target cells visualized with a fluorescent microscope.

To assay for replication-competent virus, supernatant from the putative packaging cells was harvested on Day 5 after induction and used to inoculate H9 cells. H9 cells (2×10^6) were first treated with 2 μ g/ml of Polybrene for 30 min, followed by inoculation with 0.5 ml of supernatant for 2 h. Inoculated H9 cells were continually cultured for 30 days. Supernatant samples were collected periodically, and on the 30th day used to inoculate fresh H9 cells, from which supernatant was collected 5 days later. Presence of p24^{gag} antigen was assayed in all the supernatant samples collected using HIV-1 p24 ELISA kit as described by the manufacturer (Dupont).

Southern blotting

Isolation of genomic DNA and Southern blotting analysis was performed according to standard procedures (Sambrook *et al.*, 1989). Genomic DNA (20 μ g) was digested with *SacI* and electrophoresed through a 0.8% agarose gel. Following genomic DNA transfer to a nylon membrane, the blots were probed with a ³²P-labeled 788-bp fragment of amplified DNA (corresponding to nucleotide 1261–2049 of HIV-1 RNA genome) which was obtained via polymerase chain reaction from the *gag* gene from pHIV-gpt (Yu *et al.*, 1996) (Fig. 4A).

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